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HISTOPLASMA CAPSULATUM CHITIN SYNTHASE SEQUENCES AND THEIR USE FOR DETECTION OF HISTOPLASMA CAPSULATUM AND HISTOPLASMOSIS

PRIORITY CLAIM

This application claims priority to U.S. Provisional application 60/428,135, filed November 21, 2002. The disclosure of U.S. Provisional application 60/428,135, is hereby incorporated by reference in its entirety.

FEDERAL FUNDING

The studies described herein were supported at least in part by a Merit Review administered through the Department of Veterans Affairs, a Walker Research Endowment Award, and Pilot Study Grant from the University of Arkansas. Thus, the Federal government may have rights in this invention.

FIELD OF THE INVENTION

The present invention relates generally to compositions and methods for the detection of *Histoplasma capsulatum* and the treatment of histoplasmosis.

BACKGROUND OF THE INVENTION

Histoplasma capsulatum is a dimorphic fungal pathogen capable of causing acute pulmonary disease in otherwise healthy individuals and lethal disease in immunocompromised humans (Ampel, 1996, *Emerg. Infect. Dis.*, 2: 109-116; Eissenberg, 1994, *The Interplay Between Histoplasma Capsulatum and Its Host Cells*, Vol, I, Ch. 6, W.B. Saunders Company, Ltd. London, UK; Wheat *et al.*, 1985, *Am. J. Med.*, 78: 203-210). In its most serious form, the infection disseminates throughout the body. Disseminated histoplasmosis, coinciding with laboratory evidence of HIV infection, is regarded sufficient for a diagnosis of AIDS (Castro *et al.*, 1992, *MMRW 41*: 1-14). Although AIDS currently represents the most prevalent immunocompromising disease of humans, a variety of other conditions or medical treatments can impair the human immune system and create susceptibility to diseases caused by the primary

pathogen *H. capsulatum* and associated opportunistic pathogens (Goodwin et al., 1981, Medicine (Baltimore) 60: 321-266). These predisposing conditions include advanced age, diabetes, cancer chemotherapy, or immunosuppression induced to prevent rejection of transplanted organs (Wheat *et al.*, 1982, *Ann. Intern. Med.*, 96: 159-163; Davies *et al.*, 1978, *Am. J. Med.* 64: 94-100).

In nature, *H. capsulatum* exists as a mycelium that is well-adapted for a saprotrophic mode of growth in soil (Scherr & Weaver, 1953, *Bact. Rev.* 17: 51-92). After entrance of microconidia or mycelial fragments into a mammalian host, *H. capsulatum* differentiates into budding yeast (Maresca *et al.*, 1994, *Trends Microbiol.*, 2: 110-114). In the animal host, the fungus experiences significant host-induced or environmental stress, including heat shock, exposure to higher osmolarity, changes in pH, and oxidative stress (Deepe, 1998, *J. Lab. Clin. Med.* 123: 201-205; Eissenberg & Goldman, 1994, *The Interplay Between Histoplasma Capsulatum and Its Host Cells*, Vol, I, Ch. 6, W.B. Saunders Company, Ltd., London, UK; Newman, 1999, *Trends Microbiol.*, 7: 67-71). The ability to resist or overcome environmental or host-induced stress is likely to be important for continued growth and virulence of *H. capsulatum*. In addition, host-induced or environmental stress may trigger changes in gene expression necessary for virulence.

Most fungi share considerable similarities at the nucleic acid and/or protein level. For example, there is considerable sequence identity for fungi rRNA at the sequence level. The ability to distinguish among various fungi may be of considerable importance clinically (Kasuga, T., *et al.*, 1999, *J. Clin Micro.*, 37: 653-663). For example, *H. capsulatum* requires different clinical treatment than other fungal pathogens (Li, R-K., *et al.*, 2000, *Antimicrobial Agents*, 44: 1734-1736; D.K. Stein and A.M. Sugar, 1989, *Diagn. Microbiol., Infect., Dis.*, 12: 221S-228S; Ampel, 1996). In the case of individuals with AIDS, it is essential that infections resulting in disseminated histoplasmosis be rapidly diagnosed so that appropriate treatment can be undertaken to obtain the most favorable outcome. Thus, there is a need to distinguish between *H. capsulatum* and other fungi.

There is also a specific need to distinguish between *H. capsulatum* and the closely related organism, *Blastomyces dermititidis*. Although *B. dermititidis* is also an aggressive

pathogen, *H. capsulatum* infection requires a different clinical treatment than infection with *B. dermititidis* (Li, R-K., *et al.*, 2000; Ampel, 1996). Previous work indicates there is a high level of genetic similarity between *H. capsulatum* and *B. dermatitis*. For example, it has been shown that antibodies raised against *H. capsulatum* M antigen cross react with a similar sized protein in *B. dermatititis* (Hamilton, A.J. *et al.*, 1990, *J. Med. Vet. Mycol.*, 28: 479-485). Therefore, there is a need to identify differences at the genomic level for the development of sequence-specific assays that will be able to differentiate these two closely related organisms.

A major structural component found in fungi and plants that is lacking from many other eukaryotic cells, is a cell wall. In fungi, the cell wall performs a complex set of function (see e.g., C.A. Munro and N.A.R. Gow, 2001, *Medical Mycology*, 39 (S1), 41-53) including providing a skeletal scaffolding where important cell surface components can be docked, as well as protecting the cell from external toxic threats. Major components of the cell wall include (1-3)-β-D-glucan and chitin. The amount of chitin found in the cell wall varies widely among various species of fungi. Little is known concerning the content and biosynthesis of the chitin component of the cell wall of *H. capsulatum*, as characterization of cell wall chitin has primarily focused on *Saccharomyces cerevisiae*.

To date, six classes of chitin synthases have been identified in fungi. Class III, V, and VI chitin synthases have been found to be unique to the filamentous fungi. Functionally, class I, II, and III synthases are believed to maintain the bulk of the housekeeping synthesis activities, while the enzymes of the remaining three classes (chitin synthases IV, V, and VI) have more specialized functions. In the case of the filamentous fungi, the class III synthases are believed to be responsible for synthesizing chitin for cell wall biogenesis which occurs during filamentous growth. In many cases, elimination of a single chitin synthase gene function results in little change in phenotype. Still, for at least some double mutants, such as in the case of the *chs2* and *chs3* double mutant in *Wangiella dermatitidis*, loss of these chitin synthase genes is associated with a significant decrease in virulence. These two chitin synthase genes are of the class I (*chs2*) and III (*chs3*) variety, respectively. Thus, multiple gene disruptions within the first three classes of chitin synthase genes is generally associated with significant

reductions in chitin content in the cell wall and can contribute to decreased levels of virulence.

Thus, there is a need for the development of methods which specifically detect *H. capsulatum* in humans. There is also a need to be able to distinguish this pathogen from other fungi, especially closely related pathogens, such as *Blastomyces dermititidis*. There is also need to distinguish a latent *H. capsulatum* infection from an ongoing case of histoplasmosis. The ability to closely monitor this disease in high risk populations will enable the development of early treatment protocols suitable for patients, such as immunosuppressed individuals, who may not be able to defend against advanced stages of infection.

SUMMARY OF THE INVENTION

The present invention is directed to the development and use of reagents for the detection of the dimorphic fungal pathogen *H. capsulatum*. Thus, the present invention recognizes the *chs1* and *chs2* genes in *H. capsulatum* are very similar and are often upregulated during a number of various stressful growth conditions. These two genes are of the class I and III varieties, respectively, like that found in *W. dermatitidis*. Additionally, the present invention recognizes that these genes have the potential to play a significant role in the pathogenesis of *H. capsulatum*.

For example, in an embodiment, the methods and reagents of the present invention employ different aspects of the biology of *H. capsulatum* chitin synthase 2 (*Hcchs2*) for the development of nucleic acid and protein-based assays. The present invention provides for both the detection of *H. capsulatum* infection, as well as the diagnosis of an active case of histoplasmosis. The methods and reagents of the present invention also provide for the differentiation of *H. capsulatum* from other fungal pathogens such as *Blastomyces dermatititis, Aspergillus nidulans, Aspergillus fumigatus, Aspergillus niger, Emericella nidulans, Neurospora crassa, Cryptococcus neoformans, Coccidioides immitis,* and *Candida albicans*.

Thus, in one embodiment, the present invention comprises methods and compositions to enable the specific detection of the *H. capsulatum* chitin synthase 2 intron sequences as a means to detect infection with the pathogen *H. capsulatum*.

For example, in one embodiment, the present invention comprises an isolated nucleic acid for detection of *H. capsulatum* comprising:

(a) SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6; or

(b) the complement of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6; or

(c) a fragment of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, or a fragment of the complement of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6 that hybridizes under highly stringent conditions to at least one *H. capsulatum* chitin synthase intron sequence.

In another aspect, the present invention comprises an isolated nucleic acid for detection of *H. capsulatum* comprising: (a) the nucleotide sequences set forth in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, or any complement thereof; (b) a nucleotide sequence having at least 70% sequence identity to any one of the sequences in (a); and (c) a fragment of any one of (a) or (b). Preferably, the intron is intron 1, and the primers comprise at least one oligonucleotide having the sequence SEQ ID NO: 7 or SEQ ID NO: 8.

The present invention also comprises a method for detecting *H. capsulatum* in a sample by detection of chitin synthase intron sequences. In an embodiment, the chitin synthase is chitin synthase 2 gene. Thus, in another aspect, the present invention comprises a method for detection of *H. capsulatum* in a sample comprising the steps of: (a) providing a sample; and (b) assaying for the presence of DNA comprising at least one intron of the *H. capsulatum* chitin synthase 2 gene in the sample, wherein the presence of chitin synthase intron DNA indicates that the sample contains *H. capsulatum*. In alternate embodiments, the method may comprise detection of *H. capsulatum* chitin synthase intron DNA by hybridization or PCR. Preferably, the intron detected is intron 1 of the chitin synthase 2 gene.

The present invention also allows for the detection of an active case of histoplasmosis. In this embodiment, the present invention relies on the discovery that *H. capsulatum* chitin synthase expression is upregulated during oxidative stress. Thus, in

one aspect, the present invention comprises a method for detecting an active case of histoplasmosis in a sample, comprising the steps of (a) providing a sample; and (b) assaying the sample for the presence of *H. capsulatum* chitin synthase mRNA, or any fragment thereof, wherein detection of *H. capsulatum* chitin synthase mRNA is associated with an active case of histoplasmosis.

The present invention also comprises kits for detection of *H. capsulatum*. Thus, in one aspect the present invention comprises a kit for detection of *H. capsulatum* comprising: (a) one or more containers comprising oligonucleotide primers or DNA probes comprising sequences which hybridize to at least one intron of the *H. capsulatum* chitin synthase 2 gene, and (b) at least one separate container comprising *H. capsulatum* DNA comprising chitin synthase intron DNA.

In another aspect, the present invention comprises a method for using molecular genetic techniques to provide a strain of *H. capsulatum* comprising reduced pathogenicity by preparing *H. capsulatum* in which chitin synthase gene expression is either repressed or the genomic sequence is altered such that production of functional chitin synthase protein is significantly reduced.

Also, in yet another embodiment, the present invention comprises small inhibitory RNAs which can prevent expression of *H. capsulatum* chitin synthase genes. In an embodiment, the chitin synthase gene is the chitin synthase 2 gene.

The foregoing focuses on the more important features of the invention in order that the detailed description which follows may be better understood and in order that the present contribution to the art may be better appreciated. There are, of course, additional features of the invention which will be described hereinafter and which will form the subject matter of the claims appended hereto. It is to be understood that the invention is not limited in its application to the specific details as set forth in the following description and figures. The invention is capable of other embodiments and of being practiced or carried out in various ways.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows the nucleotide sequence (5' to 3') for a partial cDNA for *Histoplasma capsulatum* chitin synthase 2 enzyme in accordance with an embodiment of the present invention (SEQ ID NO: 9).

FIG. 2 shows the nucleotide sequence (5' to 3') of the Histoplasma capsulatum genomic DNA (SEQ ID NO: 10) including the entire chitin synthase 2 transcribed region, with introns 1, 2, 3, 4, 5 and 6 underlined, as well as approximately 2012 bp of 5' UTR (untranscribed region) and 2269 bp 3' UTR of the chitin synthase gene in accordance with an embodiment of the present invention. The start codon at 2088 (ATG) and the stop codon (TAG) for the protein are shown as bold/italicized font. Putative start and stop nucleotides for the mRNA are shown as single nucleotides in bold font.

FIG. 3 illustrates the sequences (5' to 3') of intron 1 (SEQ ID NO: 1), intron 2 (SEQ ID NO: 2), intron 3 (SEQ ID NO: 3), intron 4 (SEQ ID NO: 4), intron 5 (SEQ ID NO: 5), and intron 6 (SEQ ID NO: 6) of the *Histoplasma capsulatum* chitin synthase 2 gene, as well as the location of each intron in the gene, in accordance with an embodiment of the present invention, wherein sequences are shown 5' to 3'.

FIG. 4. shows the sequence for *H. capsulatum* chitin synthase 2 polypeptide in accordance with an embodiment of the present invention.

FIG. 5 illustrates an alignment of *H. capsulatum* chitin synthase 2 polypeptide (Hcchs2) with chitin synthase proteins from *Coccidioides immitis* (Ci), *Aspergillus fumigatus* (Af), and *Aspergillus nidulans* (Ani). Also shown is the consensus sequence. The Multalin program using the default settings provided on the webstite (Multiple sequence alignment with hierarchical clustering, F. Corpet, 1988, *Nucl. Acids Res.* 16(22), 10881-10890; (available at URL:http://prodes.toulouse.inra.fr/multalin/multalin.html). Settings for symbol comparisons are described in S. Henikoff and J.G. Henikoff (1992, *Proc. Natl. Acad. Sci. USA*, 89, 10915-10919) using the original Blosum62 settings with a value of 4 added to each entry to be non-negative. The gap penalties (also the default settings as provided on the web site) are subtracted to the alignment score of 2 clusters each time a new gap is inserted in a cluster. The penalty is length dependent: it is the sum of "penalty at gap

opening" and of "penalty of gap extension" times the gap length; both values must be non-negative; the maximum for both values being 255. The similarity score is equal to the sum of the values of the matches (each match scored with the scoring table) less the gap penalties. The gap penalty is charged for every internal gap. By default, no penalty is charged for terminal gaps.

FIG. 6 shows the sequences of a pair of primers (SEQ ID NO: 7 and SEQ ID NO: 8) used to amplify chitin synthase 2 intron 1 in accordance with an embodiment of the present invention. The position of the primers is shown as underlined sequence in SEQ ID NO: 1.

FIG. 7 shows an agarose gel of PCR products obtained using *Hcchs2* specific primers with various genomic templates and the results of slot-blot hybridizations of this PCR product with this same genomic DNA from two strains of *H. capsulatum* (H1 and H2), two strains of *B. dermatititis* (B1 and B2), and *C. neoformans* (Cn), *Aspergillus nidulans* (Ani), and *Aspergillus niger* (Ang) probed with a radiolabeled PCR product derived from *H. capsulatum* chitin synthase 2 intron 1 in accordance with an embodiment of the present invention. Hybridization ofsequences from the first intron of the chitin synthase gene show specificity for *H. capsulatum*. Thus, panel (A) shows an agarose gel with products obtained from a PCR reaction using primers specific to the first intron of the *H. capsulatum* chitin synthase 2 gene (labeled as intron 1) and 300 ng of genomic DNA from various fungi as a template. Panel (B) shows the PCR product from (A), containing sequence from the first intron of the chitin synthase gene 2 of *H. capsulatum* G217B, hybridized to 3 ug of genomic DNA from various fungi. The DNA for (A) and (B) was obtained from : H1 - *H. capsulatum* G217B, H2 - *H. capsulatum* 2166, B1 - *B. dermititidis* Woods, B2 - *B. dermititidis* Green, Cn - *C. neoformans* H99, Ani - *A. nidulans* M139, and Ang - *A. niger* CBS 120-49.

FIG. 8 illustrates a reverse transcriptase PCR (RT-PCR) experiment showing that chitin synthase 2 sequences are detected in total RNA from *H. capsulatum*-infected macrophages in accordance with an embodiment of the present invention. Primers specific for *H. capsulatum* chitin synthase 2 were used in all amplifications. Lane (1) is a 1 kb ladder; lanes 2 and 3 are PCR products using total *H. capsulatum* RNA template without and with reverse transcriptase (RT), respectively; lanes 4 and 5 uninfected

macrophage total RNA (without and with RT, respectively); lanes 6-11 are RT-PCR performed with RNA isolated from macrophage cells infected with *H. capsulatum* for 30 min (lanes 6 and 7), 60 min (lanes 8 and 9), and 120 min (lanes 10 and 11) without and with RT, respectively. Samples were digested with DNAse I to remove any contaminating DNA. The larger band in lanes 3, 7, 9, and 11 are an 18S RNA normalizing control reaction, using primers specific to that gene within the same reaction tube as the *Hcchs2* PCR reaction. The *Hcchs2* product in lane 6, indicates incomplete Dnase I digestion of the sample. The 310 basepair *Hcchs2* PCR product is indicated.

FIG. 9 illustrates real-time PCR amplification of chitin synthase 2 sequences in samples comprising *H. capsulatum* total RNA in accordance with an embodiment of the present invention. Panel (A) shows real-time products produced at each amplification cycle and panel (B) shows the actual PCR products detected on an ethidium bromide stained agarose gel. Lanes 1 and 2 are a no-template control (i.e., no RNA); lanes 3 and 4 include *H. capsulatum* purified genomic DNA template (100 ng); and lanes 5 and 6 include total RNA (including DNA contaminate) isolated from *H. capsulatum* infected macrophage cells (250 ng). RNA samples were used as template before being digested with DNAse I to remove any contaminating DNA.

FIG. 10 illustrates expression of chitin synthase 2 in accordance with an embodiment of the present invention for *H. capsulatum* propagated under either yeast or in mycelia growth conditions using either dextrose (D) or glycerol (G) as a carbon source as shown by Northern analysis using [^{32}P]dCTP-labeled chitin synthase 2 probe.

FIG. 11 illustrates expression of chitin synthase 2 in accordance with an embodiment of the present invention, after challenge with H_2O_2 at the concentrations indicated above each lane as shown by Northern analysis using a [^{32}P]dCTP-labeled chitin synthase 2 probe.

DETAILED DESCRIPTION

The present invention relies on the discovery that intron sequences for the *H. capsulatum* chitin synthase 2 gene comprise highly specific domains, which lack significant identity with counterpart genes in other infectious pathogens and therefore, can function as specific markers for *H. capsulatum*. Thus, the invention relies on the

discovery that there is at least one very large intron in the *H. capsulatum* chitin synthase 2 gene that is lacking in all other known fungal chitin synthase 2 homologous genes. Also, the human host does not have a gene for chitin synthase. The fact that the host organism lacks this gene, and the significant size of at least intron 1 (~282 bp) makes the intron sequences highly specific reagents for detection of prior or current exposure to *H. capsulatum*. In addition, the present invention relies on the discovery that the gene for chitin synthase 2 is tightly regulated and one of several genes upregulated during pathogenesis. Thus, expression of the chitin synthase (at the mRNA or protein level) can be used as a marker of histoplasmosis.

Thus, the present invention recognizes that *H. capsulatum* chitin synthase intron sequences are useful as reagents for fungal specific hybridization or polymerase chain reaction (PCR) assays for *H. capsulatum*. For example, it is usually very difficult to distinguish *H. capsulatum* and *Blastomyces dermatititis*. However, using the primers of the present invention, *H. capsulatum* can be distinguished from *B. dermatititis* and other closely related pathogens. Also, the invention provides methods to distinguish patients who have been previously exposed to *H. capsulatum* from those patients who have an active case of histoplasmosis.

Definitions

In accordance with the present invention, conventional molecular biology, microbiology, and recombinant DNA techniques may be used that will be apparent to those skilled in the relevant art. Such techniques are explained fully in the literature (see, e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D. N. Glover ed. 1985).

Therefore, as used herein, the following terms shall have the definitions set out below.

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in either single stranded form, or a double-

stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, inter alia, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes.

DNA molecules may be identified by their nucleic acid sequences, which are generally presented in the 5' to 3' direction (as the coding strand), wherein 5' and 3' indicate the linkages formed between the 5'-hydroxy group of one nucleotide and the 3'-hydroxyl group of the next. For a coding-strand sequence presented in the 5'-3' direction, its complement (or non-coding strand) is the DNA strand which hybridizes to that sequence.

As used herein, the term "gene" shall mean a region of DNA encoding for the mRNA sequence that codes for a given protein/polypeptide along with elements regulating mRNA expression.

"Messenger RNA" or "mRNA" shall mean an RNA molecule that encodes for a polypeptide.

"DNA polymerase" shall mean an enzyme which catalyzes the polymerization of deoxyribonucleotide triphosphates to make DNA chains using a DNA template.

"Reverse transcriptase" shall mean an enzyme that catalyzes the polymerization of deoxy- or ribonucleotide triphosphates to make DNA or RNA chains using an RNA or DNA template.

"Complementary DNA" or "cDNA" shall mean the DNA molecule synthesized by polymerization of deoxyribonucleotides from an RNA template by an enzyme with reverse transcriptase activity.

An "isolated nucleic acid" is a nucleic acid the structure of which is not identical to that of any naturally occurring nucleic acid or to that of any fragment of a naturally occurring genomic nucleic acid spanning more than three separate genes. The term therefore covers, for example, (a) a DNA which has the sequence of part of a naturally occurring genomic DNA molecule but is not flanked by both of the coding sequences that flank that part of the molecule in the genome of the organism in which it naturally occurs; (b) a nucleic acid incorporated into a vector or into the genomic DNA of a prokaryote or eukaryote in a manner such that the resulting molecule is not identical to any naturally

occurring vector or genomic DNA; (c) a separate molecule such as a cDNA, a genomic fragment, a fragment produced by polymerase chain reaction (PCR), or a restriction fragment; and (d) a recombinant nucleotide sequence that is part of a hybrid gene, i.e., a gene encoding a fusion protein.

"Oligonucleotide", as used herein in referring to the probes or primers of the present invention, is defined as a molecule comprised of two or more deoxy- or ribonucleotides, preferably more than eight. Its exact size will depend upon many factors which, in turn, depend upon the ultimate function and use of the oligonucleotide.

"DNA fragment" includes polynucleotides and/or oligonucleotides and refers to a plurality of joined nucleotide units formed from naturally-occurring bases and cyclofuranosyl groups joined by native phosphodiester bonds. This term effectively refers to naturally-occurring species or synthetic species formed from naturally-occurring subunits. "DNA fragment" also refers to purine and pyrimidine groups and moieties which function similarly but which have non naturally-occurring portions. Thus, DNA fragments may have altered sugar moieties or inter-sugar linkages. Exemplary among these are the phosphorothioate and other sulfur containing species. They may also contain altered base units or other modifications, provided that biological activity is retained. DNA fragments may also include species that include at least some modified base forms. Thus, purines and pyrimidines other than those normally found in nature may be so employed. Similarly, modifications on the cyclofuranose portions of the nucleotide subunits may also occur as long as biological function is not eliminated by such modifications.

"Primer" shall refer to an oligonucleotide, whether occurring naturally or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product, which is complementary to a nucleic acid strand, is induced, i.e., in the presence of nucleotides and an inducing agent such as a DNA polymerase and at a suitable temperature and pH. The primer may be either single-stranded or double-stranded and must be sufficiently long to prime the synthesis of the desired extension product in the presence of the inducing agent. The exact length of the primer will depend upon many factors, including temperature, the source of primer and the method used. For example, for diagnostic

applications, depending on the complexity of the target sequence, the oligonucleotide primer typically contains 10-25 or more nucleotides, although it may contain fewer nucleotides.

The primers herein are selected to be "substantially" complementary to different strands of a particular target DNA sequence. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence or hybridize therewith and thereby form the template for the synthesis of the extension product.

As used herein, the term "hybridization" refers generally to a technique wherein denatured RNA or DNA is combined with complementary nucleic acid sequence that is either free in solution or bound to a solid phase. As recognized by one skilled in the art, complete complementarity between the two nucleic acid sequences is not a pre-requisite for hybridization to occur. The technique is ubiquitous in molecular genetics and its use centers around the identification of particular DNA or RNA sequences within complex mixtures of nucleic acids.

As used herein, "restriction endonucleases" and "restriction enzymes" shall refer to bacterial enzymes that cleave double-stranded DNA at or near a specific nucleotide sequence.

A polypeptide refers to any peptide generated from a protein or the full-length protein itself. A polypeptide may include the full-length protein or a fragment generated by proteolytic cleavage, chemical cleavage, or other means.

As used herein, the singular forms "a", "an" and "the" include plural references unless the context clearly dictates otherwise.

Unless specifically noted otherwise, nucleotide sequences are presented 5' to 3'.

Detection of Chitin Synthase Intron Sequences

The present invention is directed to nucleic acid sequences that hybridize to the introns of the *H. capsulatum* chitin synthase gene and the use of these sequences for the detection of *H. capsulatum*. The invention captitalizes on the lack of chitin synthase sequences in the human host, as well as the specificity of chitin synthase intron sequences to distinguish *H. capsulatum* from other closely-related fungi. In this way, the chitin synthase gene sequence provides a unique and specific probe for detection of *H. capsulatum* and/or histoplasmosis.

Plants and fungi share the common and unique morphology of a cell wall, although the cell walls of plants are quite different than those of fungi. While cellulose is the major component of plant cell walls, the major component of fungal cell walls are β glucans. The second most common component is chitin.

The cell wall of fungi has several functions such as, protection from the extracellular environment, stability, support for surface located enzymatic activity, and to act as a selective barrier to cellular toxins. Synthesis of chitin involves a complex pathway composed of at least three chitin synthase enzymes. One component of this complex pathway, described here, is the chitin synthase 2 gene, a class III chitin synthase. Thus, chitin, a fibrous cellulose-like polysaccharide β-linked polymer of N-acetyl-glucosamine, acts as a major exoskeleton-scaffolding component of the fungal cell wall. Notably, animal cells (such as human tissue) do not contain chitin.

Thus, in one aspect, the present invention comprises an isolated nucleic acid for detection of *H. capsulatum* comprising:

(a) SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6; or

(b) the complement of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6; or

(c) a fragment of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, or a fragment of the complement of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6 that hybridizes under highly stringent conditions to at least one *H. capsulatum* chitin synthase 2 intron sequence.

Preferably, the fragment comprises at least 8 consecutive nucleotides of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6, or the complement of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6. In an embodiment, the fragment comprises an oligonucleotide having the nucleic acid sequence SEQ ID NO: 7 or SEQ ID NO: 8.

In another aspect, the present invention comprises an isolated nucleic acid for detection of *H. capsulatum* comprising: (a) the nucleotide sequences set forth in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, or any complements thereof; (b) a nucleotide sequence having at least 70% sequence identity to any one of the sequences in (a); and (c) a fragment of any one of (a) or (b). Percent sequence identity is calculated using computer algorithms known in the art, including BLAST, using default parameters provided for alignment of nucleotide sequences of 20 bp or larger (S. Altschul, 1990, *J. Mol. Biol.*, **215**, 403-410).

The highly specific *H. capsulatum* chitin synthase probes of the present invention can be used for detection of the pathogen in a patient. Thus, in one aspect, the present invention comprises a method for detecting *H. capsulatum* in a sample, comprising the steps of: (a) providing a sample; and (b) assaying for the presence of DNA comprising an intron of a *H. capsulatum* chitin synthase gene in said sample, wherein the presence of said chitin synthase intron DNA indicates that the sample contains *H. capsulatum*. Preferably, the intron is intron 1 of the chitin synthase 2 gene. Alternatively, other chitin synthase introns may be detected. For example, in an embodiment, intron 1 of the chitin synthase 2 gene and a second chitin synthase intron from chitin synthase 2, or one of the other *H. capsulatum* chitin synthases are detected. Or, introns from other *H. capsulatum* chitin synthase genes other than chitin synthase 2 may be detected. Also preferably, the sample is obtained from a human.

In a embodiment, the method further comprises the steps of: (a) exposing the sample under high stringency hybridization conditions to at least one isolated nucleic acid that hybridizes to at least one intron of a *H. capsulatum* chitin synthase gene; and (b) determining whether there is hybridization of the isolated nucleic acid to the sample, wherein a sample comprising *H. capsulatum* exhibits detectable hybridization and a sample lacking *H. capsulatum* does not exhibit hybridization. In an embodiment, the

chitin synthase gene comprises chitin synthase 2. Preferably, the method detects intron 1 (SEQ ID NO: 1) of the chitin synthase 2 gene. Also, preferably, the isolated nucleic acid comprises: (a) the nucleotide sequences set forth in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6, or any complement thereof; (b) a nucleotide sequence having at least 70% sequence identity to any one of the sequences in (a); and (c) a fragment of any one of (a) or (b).

In an embodiment, the method is a PCR based method. Thus, the method may comprise the steps of: (a) conducting polymerase chain reaction (PCR) amplification using at least one nucleic acid primer that hybridizes to at least one intron of a *H. capsulatum* chitin synthase gene; and (b) determining the presence or absence of the PCR product resulting from the amplification. In an embodiment, the chitin synthase gene is chitin synthase 2. Preferably, the intron is intron 1 of the chitin synthase 2 gene, and the primers comprise at least one oligonucleotide having the sequence SEQ ID NO: 7 or SEQ ID NO: 8. Also preferably, the conditions for PCR amplification are chosen so that the PCR product of interest is generated in samples comprising *H. capsulatum* but not in samples that do not contain *H. capsulatum*.

The present invention also comprises a kit for detection of *H. capsulatum* using hybridization probes and/or PCR primers. Thus, in another aspect, the present invention comprises a kit for detecting *H. capsulatum* comprising: (a) one or more containers comprising at least one oligonucleotide primer or DNA probe comprising sequences which hybridize to at least one intron of a *H. capsulatum* chitin synthase gene; and (b) at least one separate container comprising *H. capsulatum* DNA comprising chitin synthase intron DNA that hybridizes to these primers. Preferably, the intron is intron 1 of the chitin synthase 2 gene.

Chitin Synthase Sequences As Small Inhibitory RNAs

This invention also comprises methods and compositions for reducing *de novo* chitin synthase expression using small inhibitory RNAs or RNA quelling. For example, in an embodiment, a strain of *H. capsulatum* that expresses wild-type levels of the chitin synthase may be transformed with a vector containing a small direct repeat of coding sequence under the regulation of a constitutive promoter.

Thus, in an embodiment, the pWU55 telomeric vector may be used for fungal transformation and a pBlueScript vector is used to construct the inhibitory expression component. In the first step, an upstream component of the *H. capsulatum* catalase B gene, from base pairs –916 to +66 with respect to the start of transcription, is ligated with the vector via a directed cloning into the *EcoR I* and *Sal I* sites. The catalase B promoter component may be obtained by PCR using a genomic template, with the primers sequences as follows: iRCATBProm5': 5'-TTTGAATTCTGATCACTGCTTCAATGCCGAGAG-3' (SEQ ID. NO. 11) and iRCATBProm3': 5'-TTTGTCGACGGCTGGGACCCTTCTTGAG-3' (SEQ ID NO. 12). The catalase B promoter is used because it is ubiquitously active in the cell.

The 5' primer (iRCATBProm5') may be tagged with a 5' *EcoRI* site followed by an internal *BclI* site. The 3' primer (iRCATBProm3') may be tagged with a *SalI* site. Next the *Ura5* terminator sequence may be obtained by PCR, using the pBY33 vector as template (Dr. William Goldman, Washington University). The amplified sequence may then be tagged with a 3'- *SalI* sequence and an internal *BclI* sequence, a 5' *SalI* sequence, and ligated with the 3' end of the catalase B sequence using the pBS *Xho I* multiple cloning site.

Next, primers derived from the coding sequence of the gene of interest are constructed to produce a product of approximately 200 bp. For example, in an embodiment, sequences of 200 bp from any location of the chitin synthase coding region (e.g., exon 1 of chitin synthase 2) may be used. The 5' primer used for amplification may be tagged with a *Xho I* sequence and the 3' primer may be tagged with an *Apa I* site. Then, the final product may be digested with *Apa I* and allowed to ligate. This ligation will produce the inverted repeat sequence of approximately 400 bp and can then be used as template for PCR amplification. This last product is then digested with *Xho I* and then ligated with the *Sal I* digested catalase B – Ura5 pBluescript construct. The final product is amplified by PCR using the pBS construct as template with T7 and M13 reverse sequencing primers. This reaction should produce significant amounts of the construct and so can be digested with *Bcl I* and then ligated with *BamH I* digested *pWU55* vector. This resulting construct may then be used to transform the *H capsulatum* strain *ura*$^-$ 5-21. The catalase B promoter will produce a transcript of the small inverted repeat and this

small RNA stem and loop transcript will initiate the *de novo* RNA quelling system in a gene specific manner. The *H. capsulatum* Ura5 terminator primer sequences are as follows: HCURA5TERM-5', 5'-AAAAGTCGACCCAACTGCAAGTATTGTTAC-3' (SEQ ID NO. 13); HCURATERM-3': 5'-AAAAGTCGACTGATCAGGATGTGCTGTATCGCATCG-3' (SEQ ID NO: 14).

Chitin Synthase as a Marker of H. capsulatum and histoplasmosis

In an embodiment, the present invention describes the use of introns from the *H. capsulatum* chitin synthase 2 gene for detection of *H. capsulatum*. The sequences of a partial cDNA and genomic DNA sequences for the *H. capsulatum* chitin synthase gene (*Hcchs2*) are shown in FIGS. 1 and 2, as SEQ ID NOs: 9 and 10, respectively. The presence and locations of introns within the coding region of the *H. capsulatum* chitin synthase 2 gene has been determined by automated DNA sequencing of *H. capsulatum* genomic DNA clones. The coding region of the chitin synthase 2 gene is interrupted by 6 introns: intron 1 (282 bp) (SEQ ID NO: 1); intron 2 (91 bp) (SEQ ID NO: 2); intron 3 (78 bp) (SEQ ID NO: 3); intron 4 (109 bp) (SEQ ID NO: 4); intron 5 (149 bp) (SEQ ID NO: 5); and intron 6 (119 bp) (SEQ ID NO: 6). The introns are shown (sequentially, from beginning to end of the gene) as underlined sequences. The sequence of the chitin synthase 2 introns 1-6 are also shown in FIG. 3 as SEQ ID NOs: 1-6, respectively.

There is a high level of homology for the coding region of proteins from *H. capsulatum* as compared to proteins from other fungi. For example, there is a high homology found between *H. capsulatum* catalase A and catalase P proteins and catalase homologues found in other fungi. There is also a high level of sequence identity for *H. capsulatum* chitin synthase protein and chitin synthases from other fungi (FIGS. 4 and 5). However, in contrast to the conservation seen in coding regions, the nucleotide sequence for the *H. capsulatum* catalase introns are not highly conserved among related fungi (Johnson *et al., Microbiology*, 148, 1129-1142, 2002). Thus, intron sequences provide a unique tool by which to identify the presence of *H. capsulatum* DNA in a sample.

In an embodiment, the intron sequences (FIG. 3) are used as hybridization probes. Because of the high specificity of *H. capsulatum* chitin synthase intron sequences, the present invention comprises a method to distinguish *H. capsulatum* from other fungal

pathogens, or combinations of other fungal pathogens such as, but not limited to, *Blastomyces dermatititis, Aspergillus nidulans, Aspergillus fumigatis, Emericella nidulans, Neurospora crassa, Cryptococcus neoformans Coccidioides immitis,* and *Candida albicans.* Preferably, there is little to no cross-reactivity with DNA from other organisms. For example, in an embodiment, intron probes from *H. capsulatum* chitin synthase 2 detect DNA from *H. capsulatum* but not from all *B. dermatitis* or *C. neoformans.*

Thus, the invention comprises a method for detecting *H. capsulatum* in a patient, comprising the step of detecting nucleic acid sequences comprising at least one intron of a *H. capsulatum* chitin synthase gene. In an embodiment, the gene is chitin synthase 2. In an embodiment, intron 1 of the chitin synthase 2 gene is detected. For example, the method may include the steps of (a) obtaining a sample from a patient; (b) exposing the sample under high stringency hybridization conditions to at least one isolated nucleic acid that hybridizes to an intron of a *H. capsulatum* chitin synthase gene; (c) determining whether there is hybridization of the isolated nucleic acid to the patient sample; and (d) assessing the presence of *H. capsulatum* DNA in the sample, wherein a sample comprising *H. capsulatum* DNA will exhibit detectable hybridization and a sample lacking *H. capsulatum* DNA will not exhibit hybridization. In an embodiment, the chitin synthase is chitin synthase 2. Also in an embodiment, the method detects intron 1 of the chitin synthase 2 gene.

Hybridizations may be performed according to standard methods. *H. capsulatum* genomic DNA may be isolated by methods known to those in the art such as the protocols described by Woods *et al.*, (Woods, J. P., *et al.,* 1992, *Molecular Microbiology,* 6: 3603-10). DNA may be denatured and spotted on membranes or digested with restriction enzymes and electrophoresed in agarose gels for transfer by capillary blotting to a solid support (*e.g.* Hybond-N membrane; Amersham Pharmacia Biotech, Inc.) and hybridized to probes. For dot blotting or slot blotting, DNA is denatured and spotted onto a solid support membrane.

Hybridization conditions can be described as ranging from low to high stringency. Generally, highly stringent conditions refer to washing hybrids in low salt buffer at high temperatures. Hybridization may be to filter bound DNA using hybridization solutions

standard in the art such as 0.5M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS) at 65°C, and washing in 0.25 M $NaHPO_4$, 3.5% SDS followed by washing 0.1 x SSC/0.1% SDS at a temperature ranging from room temperature to 68°C depending on the length of the probe (*see e.g.* Ausubel, F.M. *et al., Short Protocols in Molecular Biology*, 4th Ed., Chapter 2, John Wiley & Sons, N.Y). For example, a high stringency wash comprises washing in 6x SSC/0.05% sodium pyrophosphate at 37°C for a 14 base oligonucleotide, or at 48°C for a 17 base oligonucleotide, or at 55°C for a 20 base oligonucleotide, or at 60°C for a 25 base oligonucleotide, or at 65°C for a nucleotide probe about 250 nucleotides in length. Nucleic acid probes may be labeled with radionucleotides by end-labeling with, for example, [γ-^{32}P]ATP, or by incorporation of radiolabeled nucleotides such as [α-^{32}P]dCTP by random primer labeling. Alternatively, probes may be labeled by incorporation of biotinylated or fluorescein labeled nucleotides, and the probe detected using antibodies to the label.

Intron sequences may also be used as primers for PCR amplification of intervening *H. capsulatum* genomic DNA. Thus, oligonucleotides defined by 5'-CACTCTTTCCTATGTATATGC-3' (SEQ ID NO: 7) and 5'-CGATTTAAGGGGCAAGTTAGC-3' (SEQ ID NO: 8) (FIG. 6) hybridize to chitin synthase 2 intron 1 under highly stringent conditions, as defined herein. Thus, in an embodiment, amplification with primers comprising chitin synthase 2 intron sequences results in detection of products from *H. capsulatum* DNA, but not from *B. dermatititis* and *C. neoformans, A. nidulans, or A. niger* (FIG. 7).

The applicability of the primer specific for *H. capsulatum* chitin synthase for detection of *H. capsulatum* in clinical samples is shown in FIG. 8, showing detection of chitin synthase sequences in macrophage cells infected with *H. capsulatum* by RT-PCR using primers specific to chitin synthase 2 exon sequences. For these experiments primers Hcchs2RT(2)5': 5'-CTACCTGTGATCCCAACGAG-3' (SEQ ID NO: 15) and Hcchs2RT(2)-3': 5'-ACGCCATCCTGGTAGATTCC-3' (SEQ ID NO: 16) were used. These primers hybridize to exon 3 of the chitin synthase 2 gene to produce a 310 bp (base pair) product. Thus, in an embodiment, primers specific for chitin synthase 2 (intron or exon sequences) are used to diagnose an active *H. capsulatum* infection. For example, in

a human host, macrophage cells or tissue infected with *H. capsulatum* may be diagnostic of histoplasmosis.

Techniques for detection of amplified sequences include gel electrophoresis of the amplified DNA and visualization of the amplified product by ethidium bromide staining. Alternatively, the amplified DNA may be labeled by incorporation of oligonucleotide primers, which have been radiolabeled and products visualized by comparison to radiolabeled size markers by gel electrophoresis. Finally, unlabeled PCR products may be separated by gel electrophoresis, transferred to a solid matrix and products identified by hybridization of a radiolabeled probe which recognizes (*i.e.* is homologous to) the amplified DNA.

The amplified DNA may also be labeled by incorporation of oligonucleotide primers which have been end-labeled with a detectable chemical moiety such as, for example, biotin or fluorescein, or by incorporation of nucleotides labeled with a detectable chemical moiety such as, for example, fluorescein-dUTP, and the like. The chemically labeled products are then detected using reagents specific for that moiety. For example, PCR may be performed using primers comprising biotinylated primers specific to intron sequences from *H. capsulatum* chitin synthase 2. The amplified DNA may then be blotted to a solid support, and detected using streptavidin labeled IgG and a secondary anti-IgG antibody labeled with an enzyme, such as alkaline phosphatase, which comprises a colorimetric reaction product. The presence of the colored product provides a non-radioactive, quantitative assay for the presence of *H. capsulatum* chitin synthase 2 DNA.

In another embodiment, the nucleic acid that hybridizes to chitin synthase 2 intron DNA is arranged as a microchip or an array. In this manner, hybridization of chitin synthase 2 specific PCR products may be detected by hybridization of the PCR product to the array, as for example, by labeling the PCR product with a moiety which comprises an electrochemical, luminescent or fluorescent signal.

In yet another embodiment, the method comprises using real-time PCR wherein the PCR product is detected by the use of fluorescent dyes to detect the biosynthesis of products (FIG. 9). Real-time PCR uses incorporation of a fluorescent label as a means to monitor the amplification of PCR product via flouescence resonance energy transfer

(FRET) (Leutenegger, C.M., *et al.*, 2001, *AIDS Res. Hum. Retroviruses*, 17: 243-251, Nadkarni, M.A., *et al.*, 2002, *Microbiology*, 148: 257-266; S.J. Wall and D.R. Edwards, 2002, *Anal., Biochem.*, 300: 269-273). Commercially available thermocyclers and probes are the LightCycler and probes from Roche Applied Science, the SmartCycler from Cepheid (Sunnyvale, CA), the GeneAmp 5700 and Prism 7700 cyclers from Applied Biosystems (Foster City, CA), the iCycler iQ from BioRad (Hercules, CA) and probes from Molecular beacons (www.molecularbeacons.com) (Cockerill, F.R., *et al.* 2002, *ASM News*, 68: 77-83). The methodology is adaptable to both PCR and RT-PCR techniques, and in many cases, results are obtained in less than 1 hour (see e.g., FIG. 9A, showing products at each amplification cycle). As shown in FIG. 9B, real-time PCR may be used to provide a rapid, and unequivocal detection of *H. capsulatum* infection. The PCR product for these experiments was again generated using primers Hcchs2RT(2)5': 5'-CTACCTGTGATCCCAACGAG-3' (SEQ ID NO: 15) and Hcchs2RT(2)-3': 5'-ACGCCATCCTGGTAGATTCC-3' (SEQ ID NO: 16) that hybridize to exon 3 of the chitin synthase 2 gene to produce a 310 bp (base pair) product.

Use of Intron Sequences to Distinguish H. capsulatum from Closely-Related Pathogens

The present invention also provides reagents which allow for distinguishing *H. capsulatum* from closely related pathogens such as *Blastomyces dermatititis*, *Aspergillus nidulans*, *Aspergillus fumigatus*, *Aspergillus niger*, *Neurospora crassa*, *Cryptococcus neoformans*, *Coccidioides immitis*, and *Candida albicans*. The assay may comprise using unique chitin synthase intron sequences as reagents in either a hybridization assay or a PCR assay. In an embodiment, sequences from the chitin synthase 2 gene are used. For example, in an embodiment, sequences from intron 1 of the chitin synthase 2 gene are used. Thus, in one aspect, the present invention comprises a method for distinguishing whether a subject has been exposed to *H. capsulatum* or at least one second pathogen comprising: (a) obtaining a sample from a subject; (b) exposing the sample under high stringency hybridization conditions to at least one isolated nucleic acid that hybridizes to at least one intron from the *H. capsulatum* chitin synthase 2 gene; (c) exposing the sample under high stringency hybridization conditions to at least one isolated nucleic acid that hybridizes to sequences from a second pathogen; (d) determining whether there is

hybridization of the *H. capsulatum* chitin synthase 2 intron sequences to the sample; (e) determining whether there is hybridization of the sequences from the second pathogen to the sample; (f) assessing the presence of *H. capsulatum* in the sample, wherein a sample comprising *H. capsulatum* exhibits detectable hybridization to the *H. capsulatum* chitin synthase 2 intron sequences and a sample lacking *H. capsulatum* does not exhibit hybridization; and (g) assessing the presence of the second pathogen in the sample, wherein a sample comprising the second pathogen exhibits detectable hybridization to the sequences derived from the second pathogen and a sample lacking the second pathogen does not exhibit hybridization.

In an embodiment, the assay distinguishes *H. capsulatum* from *Blastomyces dermatititis*. In another embodiment, the assay distinguishes *H. capsulatum* from *Aspergillus nidulans, Aspergillus fumigatus, Aspergillus niger, Emericella nidulans, Neurospora crassa, Coccidioides immitis, Cryptococcus neoformans, Candida albicans,* or combinations thereof.

In an embodiment, the method further comprises a PCR-based assay comprising the steps of: (a) performing PCR using at least one isolated oligonucleotide which specifically hybridizes to intron 1 of the *H. capsulatum* chitin synthase 2 gene under conditions such that a predetermined PCR product is generated in samples comprising *H. capsulatum* but not in samples that do not contain *H. capsulatum* and determining the presence or absence of the PCR product; and (b) performing PCR using at least one isolated oligonucleotide which specifically hybridizes to DNA from the second pathogen under conditions such that a second predetermined PCR product is generated in samples comprising the second pathogen but not in samples that do not contain the second pathogen and determining the presence or absence of the PCR product. Thus, the formation of a PCR product indicates that hybridization occurred between the intron probe and the target DNA. In an embodiment, the oligonucleotide specifically hybridizes to intron 1 of the *H. capsulatum* chitin synthase gene. Also in an embodiment, the oligonucleotide comprises at least one of SEQ ID NO: 7 or SEQ ID NO: 8.

In an embodiment, the PCR assay distinguishes *H. capsulatum* from *Blastomyces dermatititis*. In another embodiment, the assay distinguishes *H. capsulatum* from *Aspergillus nidulans, Aspergillus fumigates, Aspergillus niger, Emericella nidulans,*

Neurospora crassa, Coccidioides immitis, Cryptococcus neoformans, Candida albicans, or combinations thereof.

Chitin Synthase Polypeptides For Detection of H. capsulatum

The present invention also teaches the use of *H. capsulatum* chitin synthase polypeptide sequences for detection of *H. capsulatum*. The polypeptide sequence for *H. capsulatum* chitin synthase 2 gene is shown in FIG. 4, and an allignment of *H. capsulatum* chitin synthase 2 with chitin synthase proteins from other fungi is presented in FIG. 5. In an embodiment, assay for the chitin synthase polypeptide is effective for detection of *H. capsulatum* as there is no chitin synthase protein made by the human host. It can be seen that there is homology/identity between the chitin synthase enzymes for the various fungi. There are, however, regions of the enzyme (*i.e.*, the N-terminal sequence) that appear to be pathogen-specific.

Thus, in another aspect, the present invention comprises a method for detecting *H. capsulatum* in a sample, comprising the steps of: (a) providing a sample; and (b) assaying for the presence *H. capsulatum* chitin synthase polypeptide in said sample, wherein a sample comprising *H. capsulatum* chitin synthase polypeptide contains *H. capsulatum*. In an embodiment, the method includes the steps of: (a) preparing the sample for immunoassay; (b) conducting an immunoassay with an antibody preparation which specifically recognizes *H. capsulatum* chitin synthase polypeptide to form an immune complex; (c) detecting the presence or absence of the immune complex; and (d) determining exposure to *H. capsulatum,* wherein the immune complex comprising the *H. capsulatum* chitin synthase polypeptide is detected in samples have been infected with *H. capsulatum* but is not detected in samples who have not been infected with *H. capsulatum*. In an embodiment, the chitin synthase polypeptide is from the chitin synthase 2 gene. In an embodiment, the sample is from a human subject.

Also, in another aspect, the present invention comprises a kit for detection of *H. capsulatum* via detection of chitin synthase 2 polypeptide. Thus, in yet another aspect, the present invention comprises a kit for *H. capsulatum* detection comprising: (a) one or more containers comprising an antibody preparation that recognizes *H. capsulatum*

chitin synthase 2 polypeptide; and (b) at least one separate container comprising *H. capsulatum* chitin synthase 2 protein.

The present invention also provides an immunoassay for distinguishing *H. capsulatum* from closely related pathogens such as *C. neoformans, A. nidulans*, and *A. niger*. Thus, in another aspect, the present invention comprises a method for distinguishing whether a subject has been exposed to *H. capsulatum* or at least one second pathogen comprising the steps of: (a) obtaining a sample from the subject; (b) preparing the sample for immunoassay; (c) conducting an immunoassay with an antibody preparation which specifically recognizes *H. capsulatum* chitin synthase polypeptide; (d) conducting an immunoassay with an antibody preparation which specifically recognizes at least one polypeptide from the second pathogen; (e) detecting the presence or absence of an immune complex in steps (c) and (d); and (f) determining exposure to *H. capsulatum* or the second pathogen, wherein an immune complex comprising *H. capsulatum* chitin synthase polypeptide is detected in subjects who have been infected with *H. capsulatum*, and wherein an immune complex comprising polypeptides from the second pathogen is detected in subjects who have been infected with the second pathogen. In an embodiment, the chitin synthase polypeptide is from the chitin synthase 2 gene.

In an embodiment, the assay distinguishes patients who have been exposed to *H. capsulatum* from patients who have been exposed to *Blastomyces dermatititis*. In an embodiment, the assay distinguishes patients who have been exposed to *H. capsulatum* from patients who have been exposed to *Cryptococcus neoformans* (Cn), *Asperfillus nidulans* (Ani), and *Aspergillus niger* (Ang), *Aspergillus fumigates, Emericella nidulans, Neurospora crassa, Coccidioides immitis,* or combinations thereof.

As described herein, the formation of an immune complex involves allowing chitin synthase polypeptide to interact with a binding partner and then measuring the formation, or lack of formation, of such a complex. For example, antibodies to chitin synthase polypeptides may be used to complex the polypeptide as an antigen-antibody complex. Complex formation may be measured in solution, or by allowing the complex to bind to a solid surface. In this aspect, chitin synthase protein may be identified and quantified by methods known in the art such as, but not limited to, staining of thin

sections, immunoblot analysis, sandwich assays, solution enzyme-linked immunoassay (ELISA), radioimmunoassay (RIA), and the like.

As used herein, a carrier, solid surface, or solid phase support is a surface which is capable of immobilizing cells, cell particles or soluble proteins. The support can be washed with suitable buffers to remove non-bound components, and can be incubated with protein solutions to block non-specific binding sites. Well-known solid phase supports include glass, polypropylene, dextran, nylon, modified celluloses, polyacrylamides, and the like. Included as solid surfaces for binding reactions are microtiter wells, filter arrays, beads, dip-sticks and other suitable agents for binding assays.

Chitin synthase protein/polypeptide may be detected by immunoblotting. Immunoblotting generally comprises separation of proteins primarily by molecular weight by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transfer of the separated proteins to a membrane. Proteins of interest can then be detected by exposing the membrane to an antibody to the protein(s), and detecting the formation of immune complexes by methods standard in the art. For example, an assay suitable for the methods of the present invention is the enzyme linked immunoassay (ELISA or EIA) where an enzyme bound to an antibody reacts with a chromogenic substrate to produce a product which can be detected, as for example by spectroscopic, fluorometric, or visual means. Enzymes which can be used to label the antibody for production of a detectable signal include alkaline phosphatase, horseradish peroxidase, glucose oxidase, catalase, glucose-6-phosphate dehydrogenases, and the like.

Alternatively, binding may be measured using microtiter wells or other types of reaction vessels. For example, microtiter wells may be pre-coated with antibody to *H. capsulatum* chitin synthase 2 polypeptide and a mixture comprising radiolabeled *H. capsulatum* chitin synthase 2 polypeptide and a homogenate from the sample of interest added. In this approach, binding of radiolabeled chitin synthase 2 polypeptide to the microtiter wells is displaced in a quantitative manner by increasing amounts of chitin synthase 2 polypeptide in the sample.

Antibodies may be commercially available or may be prepared by methods standard in the art. Thus, antibodies may include, but are not limited to, polyclonal

antibodies, monoclonal antibodies (mAbs), single chain antibodies, Fab fragments, chimeric antibodies, epitope-binding fragments and the like. For example, polyclonal antibodies are a heterogeneous population of antibody molecules derived from the sera of animals immunized with the antigen of interest. Adjuvants such as Freund's (complete and incomplete), peptides, oil emulsions, lysolecithin, polyols, polyanions and the like may be used to increase the immune response.

Monoclonal antibodies are homogeneous populations of antibodies to a particular antigen, and are generally obtained by any technique which provides for production of antibody by continuous cell lines in culture. Monoclonal antibodies may be humanized, to thereby reduce interaction with unrelated epitopes by the technique of single chain antibodies (*see e.g.* U.S. Patent No. 4,946,777 and Bird, *Science* 242:423-426 (1988)).

Detection of Histoplasmosis

Only a small percentage of people with antibodies to *H. capsulatum* actually have an active case of histoplasmosis. In some cases, however, histoplasmosis can be fatal. Thus, it is important to develop a method to determine whether a patient who has been exposed to *H. capsulatum* actually has an active case of histoplasmosis. Patients having an active case of histoplasmosis mount a cellular defense against the pathogen, which includes the production of increased levels of peroxides and other oxidative agents.

In an embodiment, detecting increased *H. capsulatum* chitin synthase gene activity enables detection of organisms that are actively combating the body's defense mechanisms. For example, Northern analysis using different cDNA probes show that the *H. capsulatum* chitin synthase gene is differentially expressed during yeast phase growth or under stressful conditions induced by the host defense mechanisms.

Thus, the present invention also comprises methods to distinguish a latent *H. capsulatum* infection from active histoplasmosis. In this aspect, the present invention relies on the discovery that expression of the *H. capsulatum* chitin synthase 2 is regulated by growth conditions (FIG. 10) and markedly increased by oxidative stress (FIG. 11). Also, it has been found that H. capsulatum chitin synthase genes (Chs 1-5) may be upregulated under several other conditions known to exemplify the types of stress the organism may encounter (Table 1).

Thus, upon introduction into the host, fungi experience significant environmental and/or host-induced stress, including heat shock, exposure to higher osmolarity, change in pH, and oxidative stress (Deepe, 1998, *J. Lab. Clin. Med.* 123: 201-205; Eissenberg & Goldman, 1994, *The Interplay Between Histoplasma Capsulatum and Its Host Cells*, Vol, I, Ch. 6, W.B. Saunders Company, Ltd., London, UK; Newman, 1999, *Trends Microbiol.*, 7: 67-71). The ability to resist or overcome environmental or host-induced stress is likely to be important for continued growth and virulence of *H. capsulatum*. In addition, host-induced or environmental stress may trigger changes in gene expression necessary for virulence.

To evaluate the effect of such stress, cultures may be grown, *in vitro*, under various conditions that mimic these stressful conditions. For example, growth in the presence of paraquat and hydrogen peroxide can mimic the stress of reactive oxygen species experienced by the pathogen when entering the macrophage (the macrophage oxidative burst). Other conditions, such as altering the carbon sources of glycerol and ethanol from dextrose, can mimic the stress of growth under low carbon source availability during pathogenesis. Similarly, growth in sodium chloride can mimic conditions of osmotic shock that the pathogen may experience in the lungs during the early stages of pathogenesis. Growth at newly elevated temperatures (i.e., increasing from 37°C to 43°C) can mimic the temperature change experience upon inhalation of the organism into the lungs. Finally, growth in the presence of GSNO, an efficient donor of nitrous oxide (NO), creates conditions of *de novo* oxidative stress for the pathogen and again, mimics the conditions of the macrophage oxidative burst.

Table 1
Chitin synthase gene Expression Under Conditions of Stress

Gene	Paraquat Exposure (mM)					4% Ethanol Exposure (min)				H_2O_2 (mM)		
	1	2	4	6	13	30	60	90	120	20	50	100
Chs1	+/-	+	+	+	+	+	+	+	+	+/-	+	+
Chs2	+	+	+	0.5+	++	+	+	+	++	+/-	+	++
Chs3	+	+	+	++	++	+/-	+/-	+/-	+/-	+/-	+/-	/
Chs4	+	+	+	++	++	++	++	++	++	+	+	/
Chs5	+	+	+	++	++	+	+	+	+	+	+	/

(Table 1continued)

Gene	Growth/carbon				50 mM GSNO (min)				Heat shock (43°C)				1 M NaCl (min)			
	Mycelia		Yeast		30	60	90	120	20	40	60	80	30	60	90	120
	D	G	D	G												
Chs1	/	/	/	++	/	/	/	/	+/-	+/-	+/-	+/-	+	+	++	++
Chs2	/	/	+/-	++	+	+	/	++	++	+	+	+	+	+	++	++
Chs3	/	/	/	/	/	/	/	/	+/-	+	+	+/-	/	/	/	/
Chs4	/	/	/	/	/	/	/	/	+	+/-	+/-	+/-	+/-	+/-	+	+
Chs5	/	/	/	/	/	/	/	/	+	+	+	++	+/-	+/-	+/-	+/-

Similar to the results found for *H. capsulatum*, the chitin synthase III gene of *Wangiella dermatitidis* is differentially expressed under different growth conditions. In addition, it has been shown that the chitin synthase I and III genes contribute to virulence in *Wangiella dermititidis*. It has also been determined that expression of these two genes is essential for proper development in *Wangiella dermititidis*. Thus, similar to the situation for *Wangiella dermatitidis,* the chitin synthase is likely to be essential to or significant to *H. capsulatum* pathogenesis. For example, chitin synthase expression may act to help the organism deter antimicrobial toxins of the macrophage oxidative burst, as expression of the gene is upregulated during the parasitic growth state (FIG. 10) and after an oxidative stress (FIG. 11). Thus, an assay for the chitin synthase protein (or mRNA) provides a means to monitor an active infection with *H. capsulatum* (*i.e.* histoplasmosis).

Thus, the present invention also includes a method for detecting an active case of histoplasmosis in a patient, comprising detecting the presence of *H. capsulatum* chitin synthase polypeptide. In an embodiment, the chitin synthase polypeptide is from the chitin synthase 2 gene. Thus, in one aspect, the present invention comprises a method for detecting an active case of histoplasmosis in a subject, comprising the steps of: (a) providing a sample from a subject; and (b) assaying for the presence of *H. capsulatum* chitin synthase polypeptide in said sample, wherein detection of *H. capsulatum* chitin synthase polypeptide is associated with an active case of histoplasmosis. Preferably, the method includes the steps of (a) preparing the sample for immunoassay; (b) conducting the immunoassay with an antibody preparation which specifically recognizes *H.*

capsulatum chitin synthase polypeptide to form an immune complex; (c) detecting the presence or absence of the immune complex; and (d) determining whether the subject has an active case of histoplasmosis, wherein detection of the immune complex is associated with an active case of histoplasmosis. In an embodiment, the sample is obtained from a human. Also, as an internal control can be used. For example, an enzyme, such as *H. capsulatum* catalase P, that is constitutively expressed regardless of whether the organism is experiencing oxidative stress (Johnson *et al.*, 2002) may be used to quantitate the increase in chitin synthase due to oxidative stress.

The present invention also includes a method for detecting an active case of histoplasmosis in a patient comprising detecting the presence of *H. capsulatum* chitin synthase mRNA or any fragments thereof. Thus, in one aspect, the present invention comprises a method for detecting an active case of histoplasmosis in a sample, comprising the steps of: (a) providing a sample; and (b) assaying the sample for the presence of *H. capsulatum* chitin synthase mRNA or any fragment thereof wherein detection of *H. capsulatum* chitin synthase mRNA is associated with an active case of histoplasmosis. In an embodiment, the chitin synthase mRNA is chitin synthase 2 mRNA.

In an embodiment, the method may be a hybridization based assay. Thus, the method may include the steps of: (a) exposing the sample under high stringency conditions to at least one isolated nucleic acid that hybridizes to *H. capsulatum* chitin synthase mRNA or any fragment thereof; and (b) determining the levels of *H. capsulatum* chitin synthase A mRNA based on the amount of hybridization.

In an embodiment, the method may be a PCR based assay. Thus, in an embodiment, the method includes the steps of: (a) preparing *H. capsulatum* chitin synthase cDNA using mRNA from the sample as a template; (b) conducting PCR using primers that hybridize to the *H. capsulatum* chitin synthase cDNA; and (c) ascertaining the presence or absence of product, wherein detection of the amplification product is associated an active case of histoplasmosis.

For RNA based assays, total RNA may be used. For example, total RNA may be extracted from cultures of *H. capsulatum* yeast or mycelia as described above for FIG. 8 for detection of chitin synthase sequences. Thus, as described above, the applicability of

the primer specific for *H. capsulatum* chitin synthase for detection of *H. capsulatum* in clinical samples is shown in FIG. 8, showing detection of chitin synthase sequences in macrophage cells infected with *H. capsulatum* by RT-PCR using primers specific to chitin synthase 2 exon 1 sequences. For these experiments primers Hcchs2RT(2)5': 5'-CTACCTGTGATCCCAACGAG-3' (SEQ ID NO: 15) and Hcchs2RT(2)-3': 5'-ACGCCATCCTGGTAGATTCC-3' (SEQ ID NO: 16) were used. Thus, primers specific for chitin synthase 2 (intron or exon sequences) may be used to diagnose an active *H. capsulatum* infection. For example, in a human host, macrophage cells infected with *H. capsulatum* may be diagnostic of histoplasmosis.

Thus, in an embodiment, mRNA is used as a template to generate chitin synthase 2 cDNA. For example, for quantitation of mRNA by RT-PCR, total or poly-A^+ RNA is reverse transcribed using oligo-dT primers, wherein dT is defined as deoxythymidylate. For increased specificity, the primer may is designed with 3' end which specifically hybridizes to chitin synthase mRNA.

In yet another embodiment, real-time PCR employing either total RNA or DNA template may be used. Thus, as shown in FIG. 9, real-time PCR may be used to detect *H. capsulatum* chitin synthase sequences from DNA or total RNA of *H. capsulatum*-infected macrophages. As described above, the methodology is adaptable to both PCR and RT-PCR techniques, and in many cases, results are obtained in less than 1 hour (see e.g., FIG. 9A, showing products at each amplification cycle). As shown in FIG. 9B, real-time RT-PCR may be used to provide a rapid, and unequivocal detection of *H. capsulatum* infection.

Use of Chitin Synthase Regulation to Reduce H. Capsulatum Pathogenicity

As described herein, similar to the situation for *Wangiella dermatitidis,* the chitin synthase is likely to be essential to *H. capsulatum* pathogenesis. For example, chitin synthase expression may aid the organism in deterring antimicrobial toxins of the macrophage oxidative burst, as expression of the gene is upregulated during the parasitic growth state (FIG. 10) and after an oxidative stress (FIG. 11). Development of molecular protocols to inhibit expression of the chitin synthase may therefore be used to reduce pathogenicity.

Thus, the present invention provides a method for using molecular genetic techniques to provide a strain of *H. capsulatum* comprising reduced pathogenicity by preparing *H. capsulatum* in which chitin synthase gene expression is either repressed or altered such that production of functional chitin synthase protein is significantly reduced. Thus, in another aspect, the present invention comprises knock-out strains of *H. capsulatum* in which chitin synthase 2 protein levels are significantly reduced. In an embodiment, the chitin synthase gene may be placed under control of a repressible promoter. Alternatively, the present invention comprises production of *H. capsulatum* strains in which chitin synthase expression is permanently repressed. In yet another embodiment, the present invention comprises production of *H. capsulatum* strains comprising a disrupted chitin synthase genomic sequence. In an embodiment, the strain comprising reduce chitin synthase protein is used to provide a vaccine.

EXAMPLES

Features and advantages of the inventive concept covered by the present invention are further illustrated by the examples which follow.

Example 1: Strains

The *H. capsulatum* virulent strain G-217B (ATCC # 26032; generously provided by W.E. Goldman, Washington University) and ATCC strain 2266 was used in all experiments. Two clinical isolate strains (the Woods strain and the Green strain) of *Blastomyces dermititidis* were also used in these experiments (generously provided by Dr. Robert Bradsher, University of Arkansas Medical Sciences). *H. capsulatum* and *B. dermititidis* cultures were grown with gentle shaking at 37° in 3% glycerol (v/v) or 2% dextrose (w/v) HMM medium (Worsham, P.L. *et al., J. Med. & Veterinary Mycology*, 26:137-43). YPD (1% yeast extract, 1% bacto-peptone, and 2% glucose) was used as rich medium for growth overnight at 37°C of H99, a virulent clinical isolate of *C. neoformans* serotype A (generously provided by J.K. Lodge of St. Louis University). *Aspergillus nidulans* (strain: FGSC A4 Glasgow wild type VeA+) and *Aspergillus niger* (strain: FGSC A732 *A. niger* wild type) were obtained from the University of Kansas Fungal Genetics stock center and grown in Sabouraud's media (4% Dextrose, 1% Bacto-

Peptone) for 3 days in a shaking incubator at 37 °C. The *E. coli* DH5α strain or SOLAR strain (Stratagene, Inc.) were used for plasmid transformations.

Example 2: Isolation and characterization of chitin synthase cDNA clones and Introns

A cDNA library was generated with the Lambda Zap II cDNA library kit from Stratagene Inc., according to instructions of the manufacturer. To generate the cDNA library, polyA$^+$ mRNA was isolated from strain G-217B yeast phase cells which grown in HMM-gly medium. Hence, mRNAs for most yeast phase constitutively expressed genes, such as chitin synthase, were meant to be represented in the cDNA library. Approximately 10^6 clones were isolated from the library. This library was amplified once and stored at –70 °C in 7% (v/v) DMSO (dimethyl sulphoxide). Based on blue/white screening, more than 97% of the clones demonstrated insert sizes ranging from 0.5 to 6.4 kbp (kilobase pairs).

The *Hcchs2* cDNA was originally isolated as part of a large scale screening for *H. capsulatum* catalase A cDNA using a probe that was originally designed from degenerate primers. When analyzed by DNA sequencing, however, it was found that a few of the clones contained sequences coding for chitin synthase. Initially, the degenerate radiolabeled probe was hybridized to duplicate filter lifts of bacteriophage plaques made by the cDNA library. Plaques that showed hybridization to the probe on both copies of the filter, after both the primary screening and a rescreening, were saved.

Lambda clones with large genomic fragments containing the chitin synthase gene were isolated from a *H. capsulatum* genomic library. The library was constructed by Lofstrand Labs Limited (Gaithersburg, Maryland) from Sau 3A partial digestion of *H. capsulatum* genomic DNA using the Lambda FIX II/Xho I partial Fill-in Vector Kit (Stratagene of La Jolla, CA). The library was screened using the XL1-Blue WRA (P2) strain (Stratagene, La Jolla, CA). *H. capsulatum* chitin synthase cDNA clones were used for radiolabeled probe construction and library screening. Analysis of positive genomic clones was performed by DNA sequencing to confirm both the locations and the sequences of the introns for both genes.

A partial cDNA clone for chitin synthase 2 shown in FIG. 1 (SEQ ID NO: 9). The sequence for the chitin synthase gene, including introns, and upstream and downstream untranslated regions (SEQ ID NO: 10), was determined by automated sequencing of clones isolated from the genomic library and is shown in FIG. 2. The open reading frame for the chitin synthase 2 gene encodes a protein of 905 residues with a predicted molecular mass of 101,300 Daltons and a predicted pI of 8.85. A multiple sequence alignment of the *H. capsulatum* chitin synthase 2 with known fungal chitin synthases is shown in FIG.5.

Example 3: DNA Purification and Southern/Slot Blot Analysis

H. capsulatum genomic DNA used for the construction of a genomic library was prepared by a modification of the protocol described by Woods et al. (Woods *et al.*, 1992, *Mol. Microbiol.*, 6: 3603-3610) adapted to a 50 ml culture size.

Genomic DNA for all fungi used for Southern blot (or slot blot) analysis was isolated by a modification of the Pitkin *et al.* (Pitkin *et al.*, 1996, *Microbiology*, 142: 1557-1565). Briefly, mycelial cultures were pelleted and the fungi lysed by vortexing and extraction with CTAB extraction buffer (100 mM Tris-HCl, pH 7.5, 700 mM NaCl, 10 mM EDTA, 1% CTAB (Hexadecyltrimethylammonium bromide), 1% β-mercaptoethanol) by incubation at 65°C (30 min) followed by extraction with an equal volume of chloroform, and the DNA pelleted from the aqueous supernatant by adding an equal volume of isopropanol. Upon precipitation (5000g, 10 min) the DNA is resuspended in a small volume of water, digested with Rnase A, extracted with phenol/chloroform and ethanol precipitated.

Southern blotting was performed as described by Sambrook et al., (Sambrook, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989, Chapter 9). Digested DNA (10 μg/lane) was separated by electrophoresis through 1% agarose gels, transferred by capillary blotting to Hybond-N membrane (Amersham) and hybridized to radiolabeled probes (Sambrook et al., Chapter 9). For slot blots 3 μg DNA was denatured by boiling in 30 mM NaOH and blotted to nylon membranes with a standard vacuum slot blotter. The blot was probed with a radiolabeled probe containing the first intron of the chitin synthase 2 gene using the conditions described below.

Radiolabeled probes were constructed from PCR products containing the entire transcribed region or smaller intronic regions using the procedures described in the Random Priming Labeling Kit (Invitrogen, Carlsbad, CA). For example, a radiolabeled probe for intron 1 (FIG. 3) was constructed from PCR products containing the intron regions of the chitin synthase 2 gene using primers corresponding to SEQ ID NO: 7 and SEQ ID NO: 8 as primers. PCR reaction conditions were as follows: denaturation at 94°C for 1 minute followed by 20 three-step amplification cycles of 94°C for 20 seconds, 50°C for 20 seconds, and 74°C for 20 seconds with a final extension at 74°C for 1.5 minutes.

Both Southern and slot blot hybridizations were performed in 0.5M $NaPO_4$, pH 7 and 7% SDS Church's buffer (Church, G. M. & Gilbert, W., 1984, *Proc. Natl. Acad. Sci., USA* 81: 1991-1995) at 60°C overnight and washed once at room temperature on a shaking platform for 30 min, in a 1:1 dilution of Church's hybridization buffer with water (Church, G. M. & Gilbert, W., 1984, *Proc. Natl. Acad. Sci., USA* 81: 1991-1995).

Example 4: RT-PCR of *H. Capsulatum* Infected Macrophage Cells

Methods have been devised in order to identify yeast infected macrophage cells. Initial experiments employed mouse RAW 264.7 macrophage cells infected with *H. capsulatum*.

Murine RAW 264.7 macrophage cells are grown overnight in DMEM medium (3% Fetal Bovine Serum and activated with 1000U of gamma-Interferon) in a 30 ml tissue culture flask at a density of 1.0 $X10^6$ cells. The cells were infected with *Histoplasma capsulatum* (strain G217B) at a multiplicity of infection of 10:1 yeast to macrophage cells. The infection was allowed to proceed for 1 hour and then the infected macrophage cells were washed with PBS, to remove uningested yeast, and the macrophage removed and recovered by sedimentation at 1000xg for 1 minute at 4 °C. Total RNA was recovered by extraction according to Johnson *et. al.*, 2002, *Microbiology*, 148:1129-1142.

Total RNA (1 μg) is digested with RNase free amplification grade DNase I from Invitrogen (Carlsbad, California), according to the manufacturer's protocol. Then, 1 μg of total RNA is used as template in a reverse transcription reaction , to produce cDNA

template, using Invitrogen Superscript II according to the manufacturer's protocol. An aliquot of 0.5 µl of the RT reaction is used in a PCR reaction as follows: 1 cycle of 1 step at 74°C for 1 minute and 30 seconds followed by 40 cycles of 3 steps as follows: (i) 94°C for 30 seconds; (ii): 54°C for 20 seconds; and (iii): 74°C for 25 seconds; followed by one cycle at 74°C for 1 minute and 30 seconds. Reaction products were analyzed in a 1% agarose gel using standard laboratory methods.

Example 5: Real-Time PCR and RT-PCR

Real-Time RT-PCR was performed in an ABI Prism 7700 Sequence Detection System. The real-time RT-PCR protocol is identical to that described for the gel based RT-PCR (Example 4) with the following modifications: 2 µl of the RT reaction is used in the PCR reaction, as opposed to 0.5 µl, and the PCR reactions are performed using the Invitrogen Platinum Sybr Green qPCR Supermix (UDG).

For real-time PCR the amplification conditions were as follows: a single cycle of a single step at 50°C for 2 minutes, followed by a single cycle of a single step at 95°C for 2 minutes, which was then followed by 45 cycles of 3 steps: (i): 94°C for 30 seconds; (ii): 54°C for 20 seconds; and (iii): 74°C for 25 seconds. The final step was 1 cycle at 74°C for 1 minute and 30 seconds. Amplified products were analyzed using the Applied Biosystems Sequence Detection System Analysis Software version 1.7 supplied with the ABI 7700 Detection System.

Example 6: Regulation of *H. capsulatum* chitin synthase genes in response to oxidative stress and other types of environmental stress.

To determine if the *H. capsulatum* chitin synthase genes are differentially regulated during oxidative stress and development, the abundance of transcripts for *H. capsulatum* chitin synthase in two developmental stages (mycelia or yeast) (FIG. 10) in response to H_2O_2 challenge was examined (FIG. 11).

For Northern analysis, total RNA was extracted from cultures of strain G-217B yeast or mycelia according to a modification of the acid guanidinium thiocyanate extraction procedure of Chomczynski & Sacchi (1987). RNA samples (25 µg/lane) were

electrophoresed in formaldehyde-1% (w/v) agarose gel (Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, 1989, Cold Spring Harbor Laboratory Press, Plainview, NY) transferred by capillary blotting to Hybond-N membrane (Amersham Pharmacia Biotech Inc.) and hybridized to radiolabeled probes in the hybridization solution of Church and Gilbert (Church, G. M. & Gilbert, W., 1984, *Proc. Natl. Acad. Sci., USA* 81: 1991-1995) according to the procedure described by Johnson and Schmidt (Johnson, C. H. & Schmidt, G. W., 1993, *Plant Mol. Biol.*, 22: 645-658). Results were obtained both by autoradiography and phosphorimaging (Molecular Dynamics Storm Phosphorimager, Amersham Pharmacia Biotech Inc., Piscataway , NJ). Band intensities were determined from the Phosphorimager data and normalized to the band intensity of the small subunit rRNA in the same lane. Imagequant 5.1 software (Molecular Dynamics) was used for these measurements.

As shown in Figure 10, chitin synthase expression is up-regulated in yeast cultures grown in glycerol (G) as compared to yeast cultures grown in dextrose (D) or mycelial cultures grown in glycerol or dextrose. Also, as shown in FIG. 11, chitin synthase mRNA is upregulated upon exposure to hydrogen peroxide (H_2O_2).

The effects of varying culture conditions were assessed for five chitin synthase genes in *H. capsulatum*. For the experiments shown in Table 1, the size of the cultures were 50 ml for each experiment and grown to an OD_{600} of 3 or 4 in a New Brunswick shaking incubator at 150 RPMs at 37 °C. The concentration of paraquat that each culture was exposed to for 1.5 hours is listed above each column. In the ethanol experiment, cultures were grown in the presence of 4% ethanol for increasing time as indicated. Cultures exposed to hydrogen peroxide were grown as described for paraquat using the concentrations indicted. Cultures were grown in the presence of 50 mM GSNO for increasing amounts of time, in minutes, as indicated. To mimic heat shock, cultures were grown at an elevated temperature for increasing amounts of time as indicated. To mimic stress due to change in osmolarity, cultures were grown in the presence of 1M sodium chloride (NaCl) were grown for the times indicated.

The chitin synthase expression was also determined in cultures grown in both glycerol (3% v/v) and glucose (100 mM) until reaching a density of 4 or 5 OD_{600}. The

state of growth, mycelial or yeast, and the carbon source (glucose – D, or glycerol – G) are indicated.

The cultures were recovered as described in the methods for RT-PCR and total RNA isolated as described above. Total RNA was banded by standard 1.5% agarose denaturing gel, blotted to nylon membrane and probed with chitin synthase gene specific [P^{32}]dCTP-radiolabeled probes. Signal quantification was performed visually.

Example 7: *H. capsulatum* knock-out strain construction

In *Histoplasma capsulatum*, construction of gene knock-out strains is done by homologous gene recombination. Initially, a genomic clone of choice is isolated and the chitin synthase coding region disrupted by its replacement, in the genomic fragment, with a copy of a bacterial Hygromycin B Phosphotransferase gene under eukaryotic promoter control. This construct and the remaining upstream and downstream contiguous genomic sequence (~6kb total for chitin synthase) is ligated with the transformation vector pWU55. Subsequently, the telomeric, uracil$^+$ pWU55 construct is linearized with Pac I endonuclease and used to transform the *H. capsulatum* strain G217B ura5-23 (a uracil auxotroph strain). Positive transformants are selected on HMM (Histoplasma macrophage media) agarose plates, due to their reversion to uracil prototrophy as a result of the presence of the pWU55 vector, and the positive transformants used to isolate gene knock-out strains using a positive/negative selection process.

For production *of H. capsulatum* knock-out strains, sequences from the *Hcchs2* gene are used to construct both 5' and 3' arms that are located upstream and downstream of the chitin synthase coding region, to be used in the construction of a knock-out vector. The following sequences are used to construct the 5' and 3' arms of the knock-out construct: For the 5' arm HcCS5'UTR(5'), 5'-AAGGAATTCTCTAGACCCTTGTAACCCAATGTC-3' (SEQ ID NO: 17); and HcCS5'UTR(3'), 5'-AAGGAAAAAAGCGGCCGCCAAAACGAGAGGCTGGGTTG-3' (SEQ ID NO: 18) are used. The 5' primer is tagged with an *EcoRI* sequence and the 3' primer is tagged with a *NotI* sequence. For the 3' arm, HcCS3'UTR(5'), 5'-AAGGAAAAAAGCGGCCGCGCTGCCGCTTGGCCAACTG-3' (SEQ ID NO: 19) and

HcCS3'UTR(3'), 5'-AAGTCTAGAGACCCATCTCAGCTCTTC-3' (SEQ ID NO: 20) are used. The 5' primer is tagged with a *NotI* sequence and the 3' primer is tagged with a *Xba I* sequence.

The products from PCR amplification, using *Histoplasma capsulatum* genomic DNA as template, are 2,500 bases for the 5' arm and 2,000 bases for the 3' arm in size. The disrupted *Hcchs2* gene is constructed in the pMeca vector (Thomson, J.M., and W.A. Parrott. 1998.*Biotechniques*, **24**, 922-927).

The ligations are required to be performed in a specific sequence, due to internal restriction sites, and that sequence requires that the 3' arm be ligated with pMECA first and that ligation be followed by the appropriate digestion and ligation of the 5' arm as a second step.

One of the selectable markers used in this system is the hygromycin resistance marker. *H. capsulatum* is not resistant to the antbiotic/antimycotic hygromycin B. Therefore, this gene is useful as a marker to indicate the presence of a given gene construct in a new strain after a transformation experiment. Thus, the hygromycin gene is used as both a disrupting piece of DNA within the gene of interest, in this case the *Hcchs2* gene, and as a marker as an indicator of successful incorporation of the construct within the genome of a new *H. capsulatum* strain after transformation.

In this case the *E. coli* hygromycin resistance gene (*hph*) is fused with the constitutive promoter of the *H capsulatum* calcium binding protein and has been ligated in a vector called pMV75 (W.E. Goldman, Washington University). This vector is used as a template to produce the expressable hygromycin PCR product to ligate with the two *Hcchs2* gene arms. This creates an altered form of the *Hcchs2* gene whose coding region has been disrupted by the hygromycin gene marker.

Primers for amplification of the expressable *hph* (hygromycin resistance gene) are VM75F1: 5'-TCGAGCGGCCGCGAGTTATACTGATGTCTG-3' (SEQ ID NO: 21), and

VM75R : 5'-ATCTGCGGCCGCATTACCTCTAAACAAGTG-3' (SEQ ID NO: 22).

Finally, this reconstructed form of the *Hcchs2* gene is removed from the vector by *Xba I* digestion. The disrupted form of the gene is ligated with a *Spe I* digested reconstructed *pWU55* vector (this new form of *pWU55* is termed *pSpe50*). Upon final

construction, the knock-out construct vector is digested with *Pac I*, in order to linearize the vector to the telomeric form of DNA, and used totransform the uracil minus *H. capsulatum* strain *ura⁻ 5-21*.

The reconstructed form of the *pWU55* contains aproximately 50 bp of the *pMECA* multiple cloning site ligated at the *BamH I* site of the former vector. This new form of vector was created by PCR amplification of the *pMECA* cloning site, using the T3 and T7 sequencing primers tagged with *Bgl II* restriction sequences, and after digestion with *Bgl II* the fragments were ligated with a *BamH I* digested *pWU55* vector. This resulted in the construction of a *H. capsulatum pWU55* telomeric transformation vector with a new multiple cloning region inserted at the *BamH I* site of the vector. In short, this inserts bases 396 to 477 of the *pMECA* multiple cloning site region into the *pWU55* vector at the *BamH I* site, and results in a new vector, *pSpe50*.

As described above, this vector is to be used as the receiving vector for our disrupted version of the *Hcchs2* gene. It can then be digested with *PacI* and used to transform the uracil minus form of *H capsulatum* and this strain used to select a chitin synthase minus strain.

To isolate positive transformants, the positive/negative selection process is performed as follows. A culture of the uracil prototroph strain is grown in HMM containing 50 µg/ml uracil for approximately 3 weeks. After this selection period, the culture is spread on HMM agarose containing uracil and 5-fluoroorotic acid, a compound that is toxic to uracil prototroph strains, to select for strains that have reverted to a uracil auxotroph phenotype. During this selection for revertants, the pWU55 telomeric construct DNA may be eliminated in some cells in the initial liquid culture due to the presence of uracil in the medium. Likewise, some of the uracil auxotroph revertant strains will have replaced the functional gene with the hygromycin disrupted gene by homologous recombination, resulting in the exclusion of the rest of the pWU55 vector contents, including the uracil gene that confers the uracil prototroph phenotype. Thus, these revertant strains are hygromycin B positive (resistant strains) and URA5 gene minus (uracil auxotrophs). A number of colonies (*e.g.*, about 6 to 10), are selected and tested for hygromycin B resistance, and their genomic DNA isolated and analyzed, by PCR, to determine the presence or absence of a disrupted gene. A knock-out strain will

produce a single PCR product that is larger than the wild-type gene product, due to the replacement of the wild-type gene with the larger hygromycin containing construct.

As shown in FIG. 9, chitin synthase mRNA abundance showed a 1.5 to 2 fold increase after H_2O_2 challenge. Thus, it appears that chitin synthase is up-regulated in response to oxidative challenge. These results indicate the chitin synthase 2 gene is under stress and/or developmental regulation. Thus upregulation, during the transition from the infectious mycelia to parasitic yeast growth states, may indicate the need of increased levels of chitin for continued pathogenesis. Thus, the increase in cell wall stability associated with increased synthesis of chitin is expected to make the pathogen less permeable to the toxic products of the macrophage oxidative burst or, to allow the cell wall to act as a defensive shield for the cell membrane.

Example 8 Small Inhibitory RNAs

In these experiments, the pWU55 telomeric vector is used for fungal transformation. The pBlueScript vector was used in order to construct the inhibitory expression component. In the first step, an upstream component of the *H. capsulatum* catalase B gene, from base pairs –916 to +66 with respect to the start of transcription, was ligated with the vector via a directed cloning into the *EcoR I* and *Sal I* sites. The catalase B promoter component was obtained by PCR, using genomic template, with the primers sequences as follows: iRCATBProm5': 5'-TTTGAATTCTGATCACTGCTTCAATGCCGAGAG-3' (SEQ ID. NO. 11) and iRCATBProm3': 5'-TTTGTCGACGGCTGGGACCCTTCTTGAG-3' (SEQ ID NO. 12). The 5' primer (iRCATBProm5') was tagged with both a 5' *EcoR I* site followed by an internal *Bcl I* site. The 3' primer (iRCATBProm3') was tagged with a *Sal I* site. Next the *Ura5* terminator sequence was obtained by PCR, using the pBY33 vector as template (kindly provided by W.E. Goldman). The amplified sequence was tagged with *Sal I* sequences at each end, with an internal *Bcl I* site in the 3' primer sequence, and ligated with the 3' end of the catalase B sequence using the pBS multiple cloning *Xho I* site.

Next, primers derived from the coding sequence of the gene of interest (e.g., *Hcchs2*) are constructed to produce a product of approximately 200 bp. For example, sequences from other *H. capsulatum* genes have been used in this system. The 5' primer

used for amplification is tagged with a *Xho I* sequence and the 3' primer is tagged with an *Apa I* site. The final product is first digested with *Apa I* and then allowed to ligate. This ligation produces the inverted repeat sequence of approximately 400 bp and can then be used as template for PCR amplification. This last product is then digested with *Xho I* and then ligated with the *Sal I* digested catalase B – Ura5 pBluescript construct. The final product is amplified by PCR using the pBS construct as template with T7 and M13 reverse sequencing primers. This reaction produces significant amounts of the construct and this is digested with *Bcl I* and then ligated with *BamH I* digested *pWU55* vector. This construct is then used to transform the *H capsulatum ura*$^-$ 5-21 strain. The catalase B promoter will produce a transcript of the small inverted repeat and this small RNA stem-loop transcript will initiate the *de novo* RNA quelling system in a gene specific matter. The Ura5 terminator primer sequences are as follows -- HCURA5TERM-5': 5'-AAAAGTCGACCCAACTGCAAGTATTGTTAC-3' (SEQ ID NO. 13); HCURATERM-3': 5'-AAAAGTCGACTGATCAGGATGTGCTGTATCGCATCG-3' (SEQ ID NO: 14).

Publications referred to throughout this patent application are referred to in order to more fully describe the state of the art as known to those skilled in the art in the relevant fields as of the date of the invention described and claimed herein. It should be understood that various changes and modifications to the presently preferred embodiments described herein will be apparent to those skilled in the art. Such changes and modifications can be made without departing from the spirit and scope of the present invention and without diminishing its attendant advantages. It is therefore intended that such changes and modifications be covered by the appended claims.